

# Dre-miR-2188 Targets *Nrp2a* and Mediates Proper Intersegmental Vessel Development in Zebrafish Embryos

Ana R. Soares<sup>1</sup>, Marisa Reverendo<sup>1</sup>, Patrícia M. Pereira<sup>1</sup>, Olivier Nivelles<sup>2</sup>, Hélène Pendeville<sup>2</sup>, Ana Rita Bezerra<sup>1</sup>, Gabriela R. Moura<sup>1</sup>, Ingrid Struman<sup>2</sup>, Manuel A. S. Santos<sup>1\*</sup>

<sup>1</sup> RNA Biology Laboratory, Department of Biology & CESAM, University of Aveiro, Aveiro, Portugal, <sup>2</sup> Unit of Molecular Biology and Genetic Engineering, GIGA-Research, University of Liège, Sart Tilman, Liège, Belgium

## Abstract

**Background:** MicroRNAs (miRNAs) are a class of small RNAs that are implicated in the control of eukaryotic gene expression by binding to the 3'UTR of target mRNAs. Several algorithms have been developed for miRNA target prediction however, experimental validation is still essential for the correct identification of miRNA targets. We have recently predicted that Neuropilin2a (*Nrp2a*), a vascular endothelial growth factor receptor which is essential for normal developmental angiogenesis in zebrafish, is a dre-miR-2188 target.

**Methodology:** Here we show that dre-miR-2188 targets the 3'-untranslated region (3'UTR) of *Nrp2a* mRNA and is implicated in proper intersegmental vessel development *in vivo*. Over expression of miR-2188 in zebrafish embryos down regulates *Nrp2a* expression and results in intersegmental vessel disruption, while its silencing increases *Nrp2a* expression and intersegmental vessel sprouting. An *in vivo* GFP sensor assay based on a fusion between the GFP coding region and the *Nrp2a* 3'UTR confirms that miR-2188 binds to the 3'UTR of *Nrp2a* and inhibits protein translation.

**Conclusions:** We demonstrate that miR-2188 targets *Nrp2a* and affects intersegmental vessel development in zebrafish embryos.

**Citation:** Soares AR, Reverendo M, Pereira PM, Nivelles O, Pendeville H, et al. (2012) Dre-miR-2188 Targets *Nrp2a* and Mediates Proper Intersegmental Vessel Development in Zebrafish Embryos. PLoS ONE 7(6): e39417. doi:10.1371/journal.pone.0039417

**Editor:** Fabio Martelli, IRCCS-Policlinico San Donato, Italy

**Received:** October 27, 2011; **Accepted:** May 24, 2012; **Published:** June 22, 2012

**Copyright:** © 2012 Soares et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by Fundação para a Ciência e Tecnologia - Portugal and the European Regional Development Fund program through projects PTDC/BIA-BCM/64745/2006 and PTDC/BIA-BCM/72251/2006. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: msantos@ua.pt

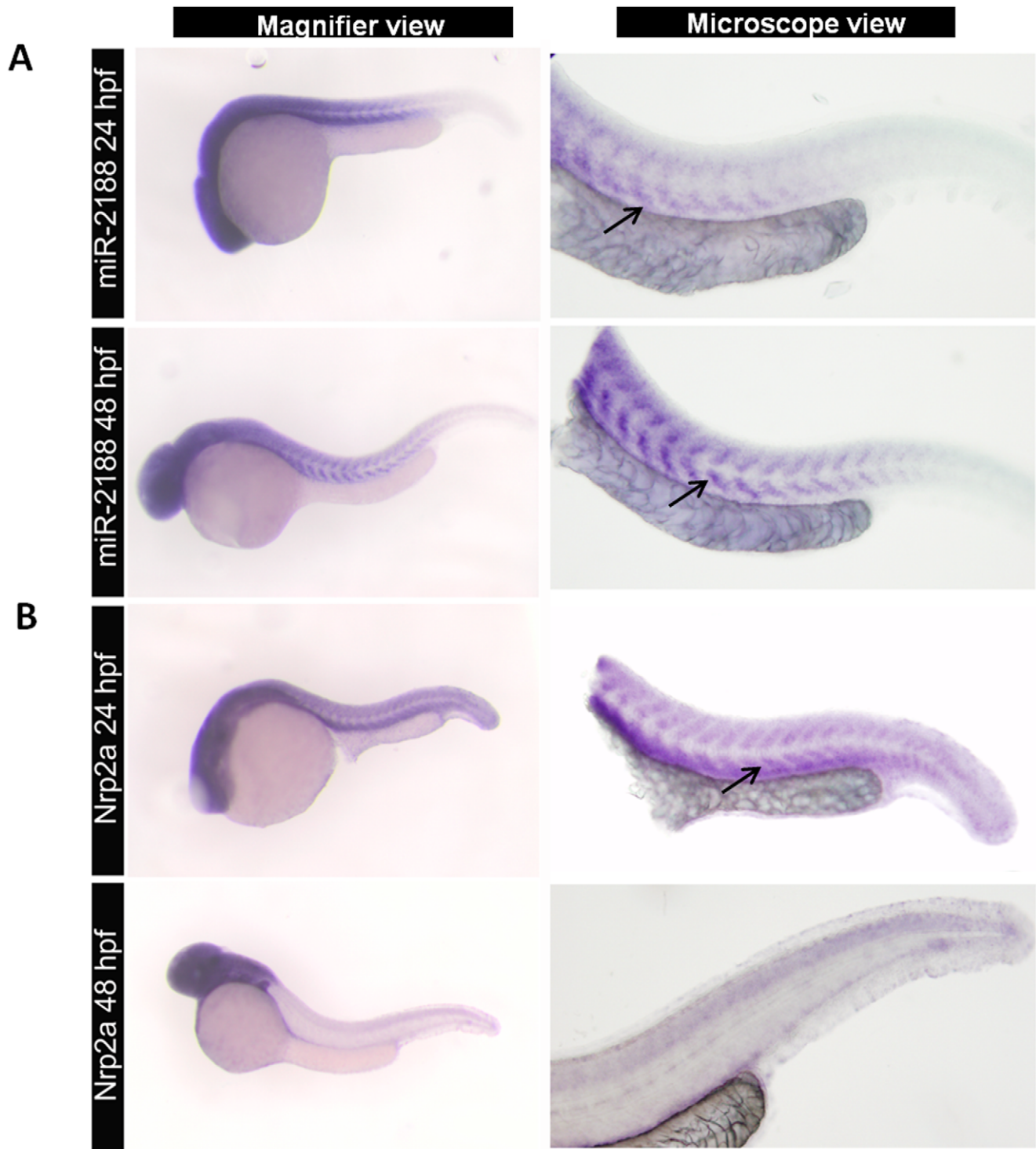
## Introduction

MicroRNAs (miRNAs) regulate gene expression at the translational level by binding to the 3'untranslated region (3'UTR) of target mRNAs and by activating their degradation or inhibiting their translation [1–4]. Eukaryotic genomes encode hundreds to thousands of miRNAs [2] and a single miRNA can regulate the expression of hundreds of genes while one gene can be regulated by more than one miRNA [1,5,6]. These molecules are implicated in the regulation of several biological processes, are important for normal development and physiology [7–11] and their deregulation is implicated in disease [12–14]. Knowledge of miRNA targets is therefore pivotal to understand the function of these small non-coding RNA molecules. Our group has recently identified novel zebrafish miRNAs and identified putative targets for some of those miRNAs [15]. Our algorithm predicted *Nrp2a*, a VEGF co-receptor involved in developmental angiogenesis, as a miR-2188 target suggesting that this miRNA may be involved in the regulation of this process.

Neuropilins (Nrp) are expressed in the vascular system with some degree of vessel type specificity in human, mouse, chicken

and zebrafish embryos [16] and mediate normal developmental angiogenesis [17–19], which is a process by which new blood vessels form in response to external stimuli sensed by vascular endothelial cells. Disruption of this process leads to abnormal vessel growth and contributes to ischemic, inflammatory and immune disorders [20]. Although Nrps are not able to initiate signaling pathways by themselves and require interaction with other co-receptors, such as the VEGFRs (VEGFR-1, VEGFR-2 and VEGFR-3) [21], their deregulation is implicated in pathological angiogenesis in tumors and retinal disease [22,23].

In zebrafish there are 4 Neuropilin genes, namely *Nrp1a*, *Nrp1b*, *Nrp2a* and *Nrp2b* [19,24]. *Nrp2a*, the predicted target of miR-2188, is an ortholog of human *Nrp2* that interacts with VEGFR2 and is required for correct development of major intersegmental vessels (ISVs) in zebrafish embryos [19,24]. Knocking down *Nrp2a* in zebrafish embryos results in arterio-venous malformations in the tail and irregular ISV patterning, while double knockdowns (*Nrp2a* and *Vegf*) increase vasculature defects and circulation arrest, which is not observed in single knockdowns [19]. In other words, there is physiological interdependence between *Nrp2a* and *Vegf* during embryonic



**Figure 1. Expression of miR-2188 and *Nrp2a* in zebrafish embryos.** A) ISH of miR-2188 at 24 hpf showed low expression on the trunk and tail, however it increased at 48 hpf between the somite boundaries. Arrows indicate hybridization signal. B) *Nrp2a* ISH at 24 and 48 pf. At 24hpf *Nrp2a* was predominantly expressed in the head and somitic regions, while at 48 hpf its expression was restricted to the brain and rombencephalon. There was an indirect correlation between miR-2188 and *Nrp2a* expression. Increasing miR-2188 levels through development were accompanied by decreasing *Nrp2a* expression between the somite boundaries. All embryos are in lateral view, anterior to left. Microscopic pictures were taken with 6,3x magnification.

doi:10.1371/journal.pone.0039417.g001

development and *Nrp2a* is likely involved in angiogenesis. Indeed, over expression of *Nrp2*, the human ortholog of *Nrp2a*, enhances vessel proliferation and migration induced by VEGF,

suggesting that *Nrp2* increases the response to VEGF and plays an important role in vessel proliferation [25]. However, little is known about the regulation of *Nrp2a* by miRNAs.

Several studies implicate miRNAs in the regulation of angiogenesis. The miRNAs implicated in angiogenesis can be classified as pro-angiomiRs and anti-angiomiRs. The former promotes angiogenesis by targeting negative regulators of angiogenesis and the latter inhibits angiogenesis by targeting positive regulators of angiogenesis [26]. For instance, miR-92a (miR-17~92 cluster) is expressed in vascular endothelial cells and suppresses the function of pro-angiogenic proteins by inhibiting translation of their mRNAs [27]. In zebrafish embryos, over expression of this anti-angiomiR causes severe defects in vessel formation [27]. Conversely, miR-126 is a pro-angiomiR and promotes VEGF signaling by inhibiting the negative regulators of the VEGF pathway, i.e., *Spred1* and *Pik3r2* [28,29]. This miRNA is required for the maintenance of vascular structure *in vivo* in mice and in zebrafish and works as an endothelial cell-specific regulator of angiogenic signaling. Deletion of miR-126 causes loss of vascular integrity which is characterized by leaky vessels, hemorrhage and partial embryonic lethality in mice [29]. In zebrafish, knockdown of this miRNA also causes hemorrhage and collapse of the dorsal aorta and primary cardinal veins, indicating that miR-126 function is conserved in vertebrates [28]. miR-296 is also a pro-angiomiR that promotes angiogenesis *in vitro* [30] and injection of a miR-296 antagomir inhibits glioma angiogenesis *in vivo*, supporting a role for this miRNA in promoting angiogenesis in tumors [26]. These findings indicate therefore that the VEGF pathway and angiogenesis can be regulated at different levels by miRNAs, suggesting that elucidation of the biology of angiomiRs is fundamental to understand angiogenesis.

Here, we show that dre-miR-2188 targets *Nrp2a* and has an important role in modulating ISV development in zebrafish embryos. Over expression of miR-2188 in Tg(flk1-GFP)<sup>s843</sup> zebrafish embryos, in which GFP is expressed in vascular endothelial cells under the control of *Flk* (*VEGFR2*), down regulates *Nrp2a*. This down regulation is accompanied by thinner and underdeveloped ISVs, without through connections with the dorsal longitudinal anastomotic vessel (DLAV). In some cases, lack of one or more ISVs is observed. We also validate *Nrp2a* as an *in vivo* target of miR-2188 demonstrating that miR-2188 modulates the expression of a positive regulator of angiogenesis. This is the first report of control of *Nrp2a* by a miRNA and indicates that the development of proper ISVs during development is dependent on the regulation of *Nrp2a* levels by a miR-2188 in zebrafish.

## Results

### miR-2188 Inhibits *Nrp2a* Expression

We have identified the zebrafish miR-2188 in a previous study by deep DNA sequencing [15] and have used computational tools to predict its putative targets. This analysis showed that miR-2188 has two putative binding sites in the 3'UTR of *Nrp2a* (Table 1). In order to experimentally validate this prediction, we have analyzed miR-2188 and *Nrp2a* expression in zebrafish embryos by *in situ* hybridization (ISH). Since miRNAs regulate gene expression, a negative correlation between miRNA and target expression is generally observed. In other words, the increase of miR-2188 expression in specific tissues should be accompanied by a decrease in *Nrp2a* expression. MiR-2188 was detected at low levels at 24 hpf, but its expression increased at 48 hpf throughout the embryo tail (Figure 1-A). On the other hand, *Nrp2a* was widely expressed at 24 hpf but its expression became restricted to the brain and rombencephalon after 48 hpf, confirming previous studies (Figure 1-B) [19,24].

To test whether *Nrp2a* was a direct target of miR-2188, we have induced miR-2188 deregulation by injecting a synthetic miR-2188

**Table 1.** Putative binding sites of miR-2188 in *Nrp2a* 3'UTR.

<i>Nrp2a</i> predicted binding sites	Match	Mfe (kcal/mol)
<b>Binding site-1 (BS-1)</b>	target 5' G G C U C C 3' GGA AU UG GG TGGACCUU CCU UA AC CC ACCUGGAA miRNA 3' G C U A 5'	−26,9
<b>Binding site-2 (BS-2)</b>	target 5' U AAC A A 3' GCA GGGUU GGACCU UGU UCCAA CCUGGA miRNA 3' CC ACAC A 5'	−22,1

doi:10.1371/journal.pone.0039417.t001

duplex to over express it or a morpholino (MO) to inhibit it, in one cell zebrafish embryos (Figure 2-A). qPCR quantification showed that over expression of miR-2188 decreased expression of *Nrp2a*, whereas miR-2188 knockdown increased *Nrp2a* expression in 48 hpf embryos (Figure 2-B, C). ISH confirmed qPCR data as there was a decrease in *Nrp2a* signal upon miR-2188 over expression when compared with scrambled duplex injected embryos at both 24 hpf (Figure 3– A-D) and 48 hpf (Figure 3– I-L). Moreover, there was an increase in *Nrp2a* signal between the somite boundaries after injection of the MO<sub>miR-2188</sub> at both 24 hpf (Figure 3– E- H) and 48 hpf (Figure 3– M-P)). These results are consistent with the target/anti-target theory which postulates that miRNAs and their targets are expressed in a non-overlapping manner, either spatially or temporally [31].

To further test if *Nrp2a* was a true target of miR-2188, we have constructed a chimeric sensor assay based on a fusion between the GFP coding region and the 3'UTR of *Nrp2a*, which contained the putative miR-2188 binding sites or mutated miR-2188 binding sites (Figure 4-A). As expected, there was a strong down regulation of GFP levels in the miR-2188 injected embryos (Figure 4-B, C). On the other hand, binding site-1 mutation (M1), but not binding site-2 mutation (M2), in the 3'UTR of *Nrp2a* abolished the inhibitory activity of miR-2188 on GFP expression (Figure 4-C), demonstrating that *Nrp2a* is targeted *in vivo* by miR-2188 and that its down regulation is mediated by a single binding site (binding site –1) present in its 3'UTR.

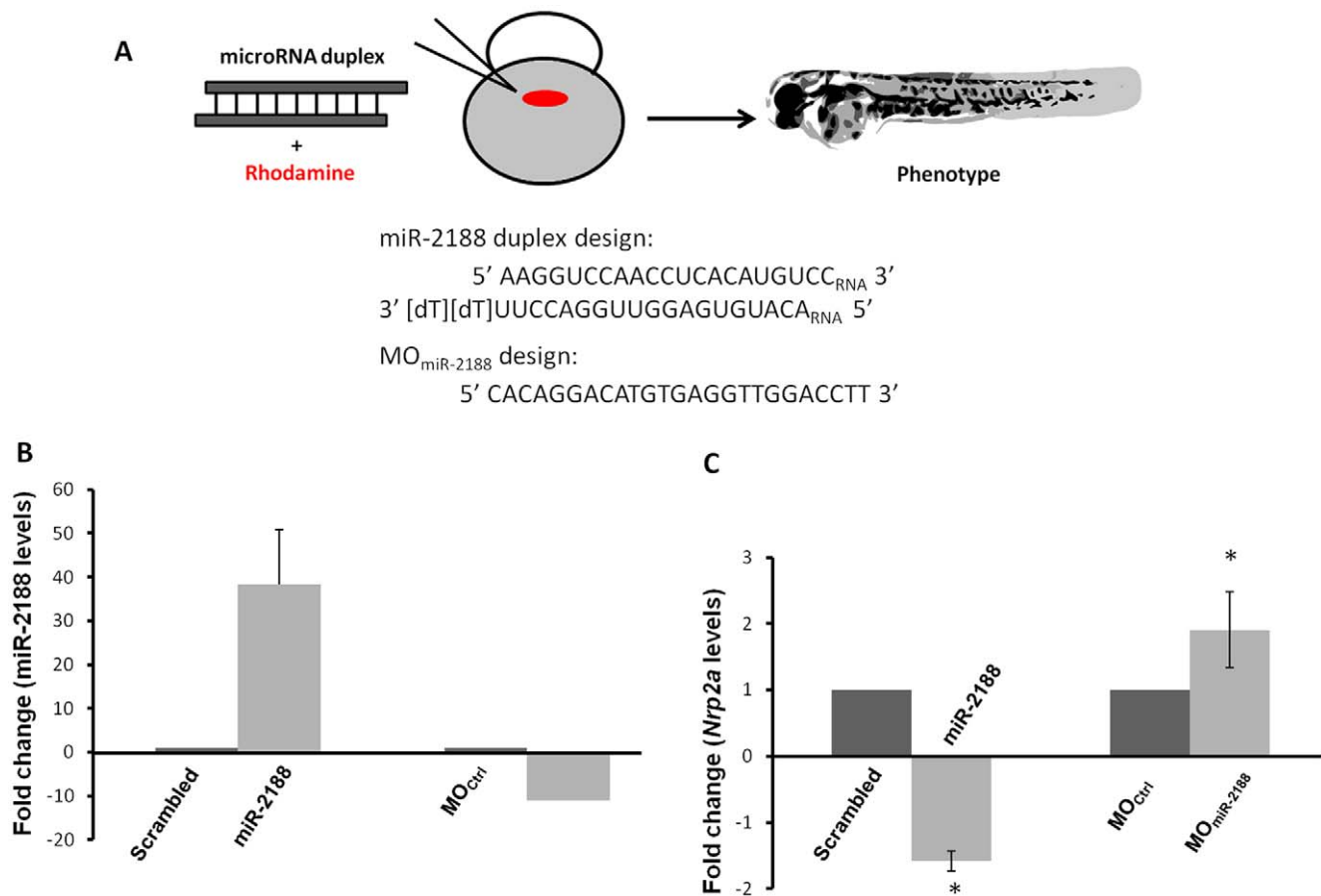
To further confirm that *Nrp2a* is a bona fide target of miR-2188, we have performed rescue experiments where a MO<sub>miR-2188</sub> was injected in the presence of the miR-2188 duplex (Figure 5). Co-injection of the MO<sub>miR-2188</sub> and miR-2188 duplex resulted in the recovery of the GFP expression, as observed both by fluorescence microscopy (Figure 5-A) and western blotting (Figure 5-B), confirming that *Nrp2a* is a miR-2188 target. Moreover, the injection of a scrambled miRNA sequence had no effect on the fluorescence levels and there was a slight increase of GFP expression on miR-2188 morphants, as expected (Figure 5).

### miR-2188 Affects Intersegmental Vessel Development

Since miR-2188 targets *Nrp2a*, which is a VEGF co-receptor required for the correct development of major ISVs, we expected disruption of this process in miR-2188 deregulated embryos. To test this hypothesis we have used transgenic zebrafish embryos expressing GFP in vascular endothelial cells under the control of *Flk* [Tg(flk1-GFP)<sup>s843</sup>].

Nrp2 family members interact with FLK (VEGFR2) and with other co-receptors to initiate the signaling pathways [21], thus it is expectable that alteration in FLK levels is observed in transgenic embryos after miR-2188 over expression. Previous studies have





**Figure 2. miR-2188 duplex and morpholino injections.** A) A miRNA duplex and a MO<sub>miR-2188</sub> were designed to over express and knockdown miR-2188 in zebrafish embryos, respectively. Duplex microinjections were performed in one cell stage embryos with rhodamine to visualize the injection process. Approximately 1000 pL of each duplex were injected. 2  $\mu$ M of each duplex were chosen as the working concentration for the injections, as this duplex concentration did not induce high mortality rates or unspecific side effects. B) qPCR quantification of miR-2188 showed that miR-2188 duplex injection increased miR-2188 expression by 40 fold and MO<sub>miR-2188</sub> injection decreased miR-2188 expression by 12 fold. C) qPCR quantification of *Nrp2a* levels in 48 hpf embryos after over expression and knockdown of miR-2188 showed that this target was down regulated 1.6 fold after miR-2188 over expression and was up regulated 2 fold after miR-2188 knockdown.  
doi:10.1371/journal.pone.0039417.g002

shown that knocking down *Nrp2a* resulted in arteriovenous malformations in the tail and irregular ISV patterning in zebrafish embryos [19]. A similar phenotype was induced by miR-2188 over expression (Figure 6, Figure S1). A delay or even absence of ISV formation was observed at 24 hpf, likely due to a decrease in *Nrp2a* expression caused by miR-2188 over expression (Figure 6–B). This was confirmed at 48 hpf when ISVs were already formed and differences in vessel development could be observed by confocal microscopy (Figure 6 - C-E). Control embryos (uninjected and scrambled injected embryos) had fully developed and thick ISVs whereas miR-2188 injected embryos had thinner, underdeveloped ISVs, with abrupt interruptions, lacking complete connections with the dorsal vessel (Figure 6-B-D). On the other hand, injection of the MO<sub>miR-2188</sub> induced ISV branching that often resulted in DLAV defects (Figure 6-C, E). Therefore, over expression and knocking down of miR-2188 affected angiogenesis differentially; supporting the hypothesis that deregulation of miR-2188 interferes with ISV development by regulating *Nrp2a* expression.

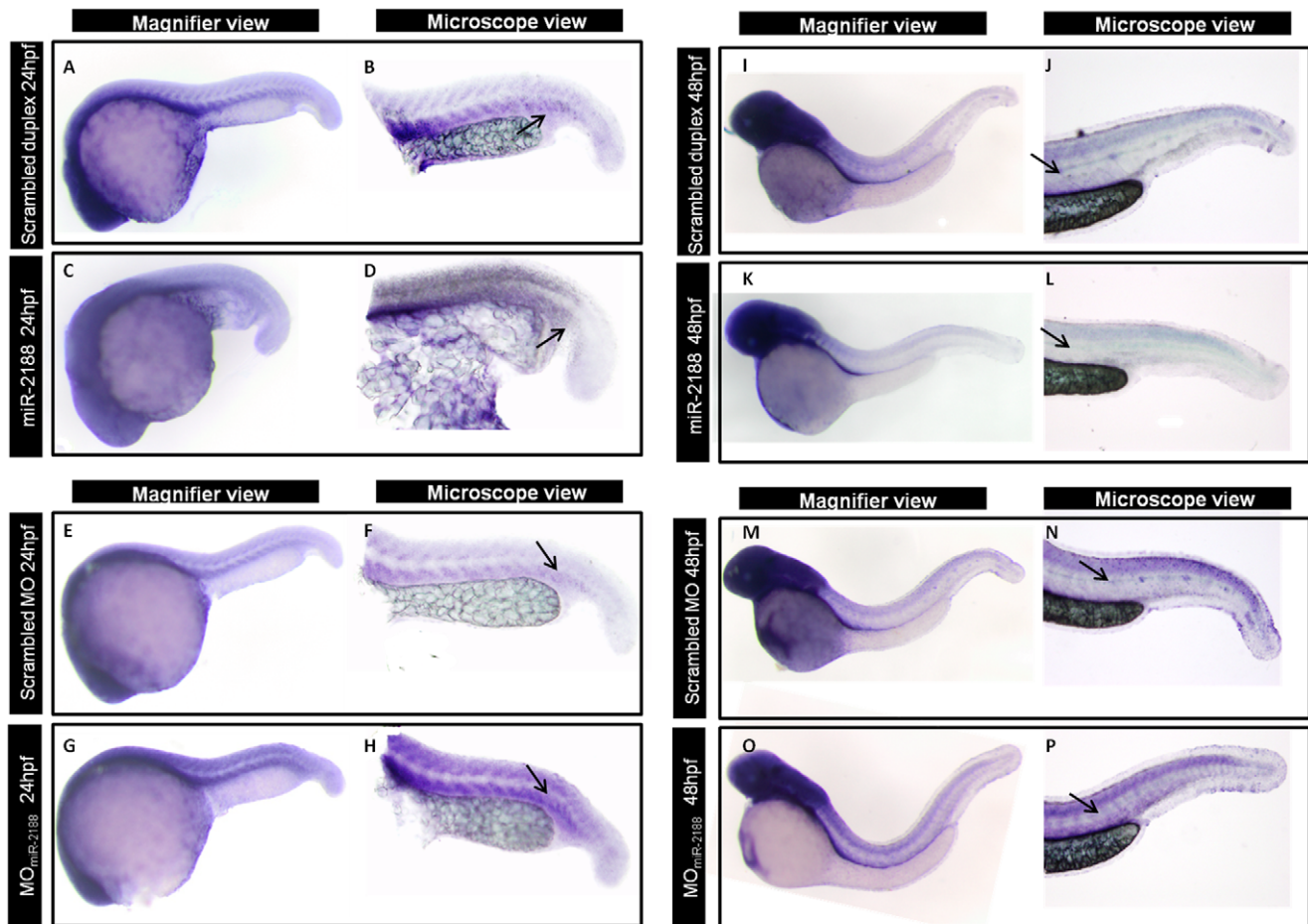
At 48 hpf 50% of the miR-2188 injected embryos displayed circulation arrest and 70% had pericardial edema (Figure S1). Absence of one or more ISVs was observed in 60% of the miR-2188 injected embryos (Figure S1). These results indicated that miR-2188 affected angiogenesis in zebrafish embryos and were

further substantiated by the injection of a MO<sub>miR-2188</sub> that blocked processing and inhibited the activity of this miRNA. MO<sub>miR-2188</sub> injected embryos were mainly characterized by irregular development of ISVs characterized by branching that often resulted in DLAV defects when compared to control embryos (60%; Figure S1; Figure 6–C, E). This phenotype was different from the phenotype obtained after miR-2188 over expression, indicating that over expression and knockdown of this miRNA affected angiogenesis distinctly, supporting the hypothesis that deregulation of miR-2188 interferes with angiogenesis.

This hypothesis was further confirmed by rescue experiments where MO<sub>miR-2188</sub> was injected in embryos overexpressing miR-2188. Co-injection of MO<sub>miR-2188</sub> with the miR-2188 duplex rescued ISV patterning, indicating that de-regulated levels of miR-2188 induce ISV defects (Figure S2).

## Discussion

Experimental validation of miRNA targets is essential to understand the functions of these small RNA molecules. Experimental validation of predicted targets is often achieved by miRNA gain or loss-of-function followed by monitoring mRNA and protein levels of the predicted targets, or by monitoring the activity



**Figure 3. ISH of *Nrp2a* after miR-2188 deregulation.** A) After injection of miR-2188 duplex, *Nrp2a* detection decreased between the somite boundaries at 24 hpf. On the other hand, in miR-2188 morphants, *Nrp2a* detection was more pronounced throughout the trunk and tail region, between the somite boundaries, when compared with the control. B) At 48 hpf and after miR-2188 overexpression, *Nrp2a* was not detected in the blood vessels or in the PCV when compared with the control embryos. On the other hand, miR-2188 morphants express *Nrp2a* in the trunk and tail, especially between the somite boundaries, which is not detected in 48 hpf control embryos. All embryos are in lateral view, anterior to left. Arrows indicate hybridization signal. Microscopic pictures were taken with a 6,3x magnification.  
doi:10.1371/journal.pone.0039417.g003

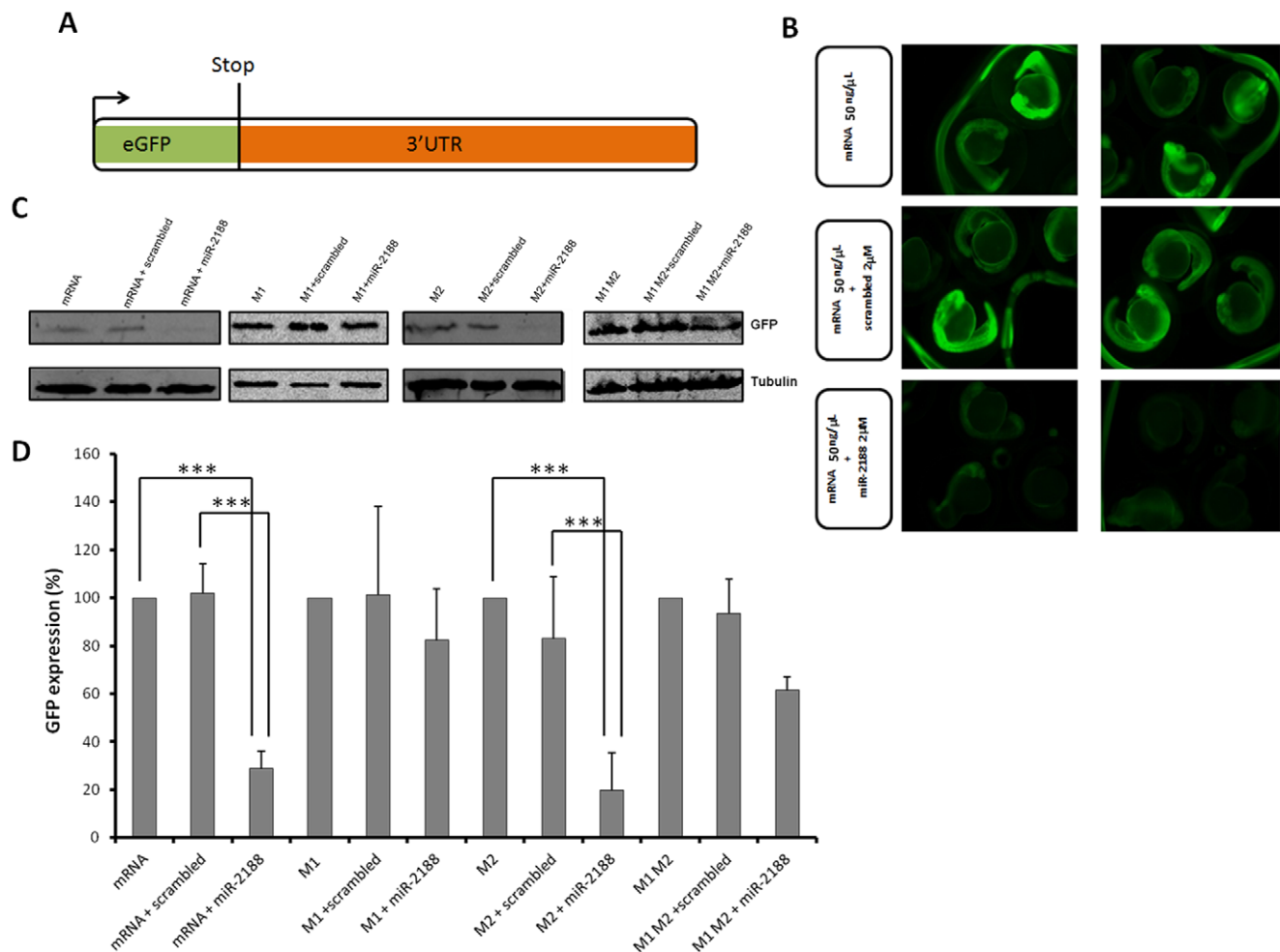
of a reporter gene whose mRNA 3'UTR contains miRNA target sites [32,33].

In this study, we have experimentally validated the miR-2188 target prediction obtained in a previous study [15]. The data show that miR-2188 regulates *Nrp2a*, a VEGF co-receptor, which is required for proper development of major ISVs in zebrafish [19]. Neuropilins increase the response to VEGF and are up regulated in endothelial tumor cells [25]. The identification of a miRNA that regulates vessel development and integrity by targeting a VEGF receptor has therefore implications for vascular development.

We have demonstrated that the *Nrp2a* 3'UTR is targeted by miR-2188 in zebrafish embryos and that this miRNA interferes with ISV development. Its over expression produced a similar phenotype to that induced by a *Nrp2a* MO, supporting the hypothesis that miR-2188 is implicated in angiogenesis by regulating *Nrp2a* expression [19]. As expected, *Nrp2a* levels are down regulated by miR-2188 over expression during early developmental stages, which is incompatible with normal ISV development in zebrafish. A decrease of *Nrp2a* expression is observed in 24 and 48 hpf embryos injected with the miR-2188 duplex, supporting the hypothesis that miR-2188 regulates this VEGF co-receptor. Conversely, knocking down miR-2188 with a

MO<sub>miR-2188</sub>, results in *Nrp2a* up regulation at 24 and 48 hpf. Interestingly, at 48 hpf the increase in *Nrp2a* expression is observed between the somite boundaries and in the DA and PCV, regions where *Nrp2a* is barely expressed in normal conditions. This indicates that knocking down miR-2188 increases *Nrp2a* expression, which is likely responsible for the irregular ISV patterning that often results in DLAV defects. This phenotype is distinct from that obtained in embryos over expressing miR-2188, supporting the hypothesis that deregulation of the latter interferes with ISV patterning. Moreover, proper ISV patterning is rescued by injection of MO<sub>miR-2188</sub> in embryos over expressing miR-2188.

Our data shows that miR-2188 interferes with proper ISV development by directly targeting *Nrp2a*, indicating that angiogenesis can be regulated at the level of VEGF receptors by miRNAs. One cannot exclude that this miRNA may also target other mRNAs involved in the VEGF pathway. Indeed, miRNAs can be master regulators of key pathways by targeting multiple mRNAs in a coordinated fashion [28,33–35]. For example, miR-126 targets *Spred1* and *Pik3r2* genes, which are involved in the VEGF pathway showing that angiogenesis and vascular integrity can be disrupted by a single miRNA [28,29]. Also, the neuronal enriched miR-128 regulates *Reelin* and *Dcx*



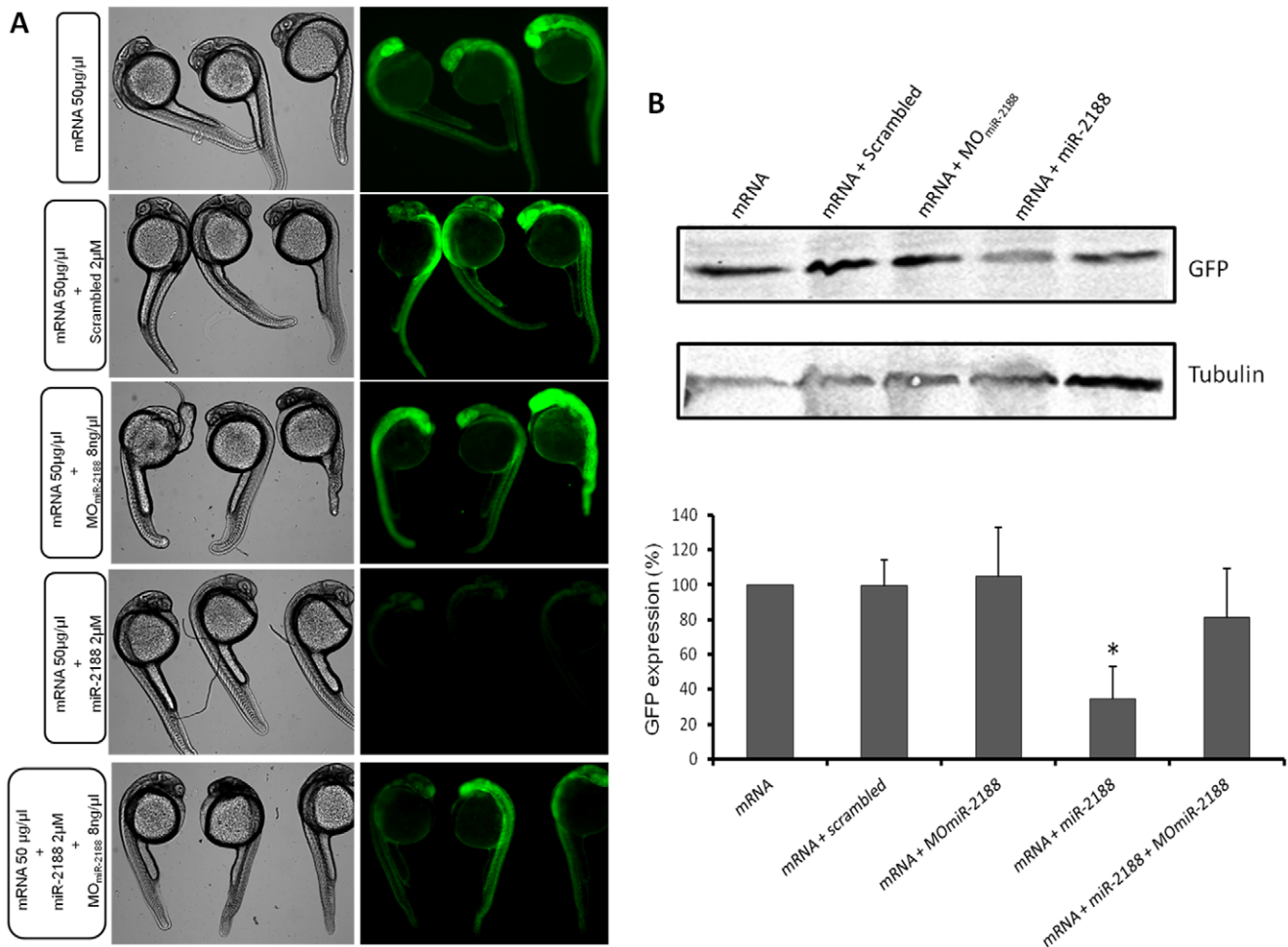
**Figure 4. GFP sensor assay.** A) The GFP gene was fused with the 3'UTR of *Nrp2a* that contained the putative binding sites of miR-2188. This construct was transcribed *in vitro* into capped mRNA prior to injection. B) Single cell embryos were injected with the GFP reporter (50 ng/μL) in the presence or absence of miR-2188 and scrambled duplexes. Fluorescence levels were observed at 24 hpf and representative embryos of each condition were photographed. Decreased fluorescence was observed in miR-2188 duplex injected embryos. C) Embryo lysates were prepared from 24 hpf embryos injected with the wild type GFP reporter or the mutated reporters in the presence or absence of miR-2188 and scrambled duplexes. Protein levels were determined by western blot analysis and β-tubulin was used as an internal control. D) Quantification of the reporter proteins (%) in the conditions tested using 3 biological replicates. Asterisks indicate conditions where GFP expression was significantly down regulated by miR-2188 relative to control conditions. miR-2188 injected embryos showed a statistically significant down regulation of GFP levels, indicating that *Nrp2a* 3'UTR is a true miR-2188 target. Co-injection of the M1 reporter with the miR-2188 duplex produced normal GFP levels, indicating that the BS-1 mutation abolished miR-2188 binding. On the other hand, a significant decrease in GFP expression was observed after co-injection of the M2 reporter with miR-2188, relative to the controls, indicating that BS-2 was not critical for miR-2188 binding. Data are mean ± stdev,  $p < 0.005$  (t test, unpaired),  $n > 3$ . All lanes were normalized to the β-tubulin signal. doi:10.1371/journal.pone.0039417.g004

and migratory potential of neuronal cells through distinct mechanisms [35], while miR-9 regulates the Fgf signaling by inhibiting *Fgf3*, *Fgfr1* and *Canopy1* and exerts a proneurogenesis effect by inhibiting expression of antineurogenic bHLH [33]. Therefore, one should not exclude the hypothesis that miR-2188 has multiple targets, which were not detected due to the high stringency of our initial prediction method. To clarify this issue, we have repeated the target prediction analysis for miR-2188 as before [15], by applying a lower stringent cutoff (with perfect seed match between nucleotides 2 and 7 and allowing up to 7 mismatches in the remaining sequence). This methodology was able to retrieve 8 additional putative targets for miR-2188, namely *Nfe2l1*, *Has2*, *Myst3*, *Cpla2*, *Atg13*, *Dnajc3*, *Dnaja3a* and *Pdik1l* (Table 2). Most of the predicted target genes are related with protein folding and heat shock response (*Atg13*,

*Dnajc3* and *Dnaja3a*) and are necessary for the proper embryonic development (*Myst3*). Interestingly, *Cpla2* is involved in the VEGF signaling pathway and regulates endothelial cell cycle progression and angiogenesis [36]. In fact, it has been shown that this gene promotes the angiogenic tubule formation as its inhibition results in defects in the endothelial cell proliferation machinery [36]. Moreover, this gene is highly expressed in several carcinomas and is associated with tumor angiogenesis [37]. Due to the involvement of this putative target in angiogenesis, similarly to *Nrp2a*, future work should validate *Cpla2* as a miR-2188 target.

Nrp genes are widely conserved among vertebrate species. For example, human *Nrp2* and zebrafish *Nrp2a* orthologs have 57% similarity [19], but miR-2188 has not been identified to date in humans [38], suggesting that regulation of *Nrp2a* by miR-2188 may





**Figure 5. GFP rescue sensor assay.** A) Single cell embryos were injected with the GFP reporter (50 ng/μL) in the presence or absence of MO<sub>miR-2188</sub>, miR-2188 and scrambled duplexes or a mixture of both MO<sub>miR-2188</sub> and miR-2188 duplex. Fluorescence levels were observed at 24 hpf and representative embryos of each condition were photographed. Fluorescence was recovered in MO<sub>miR-2188</sub>+ miR-2188 injected embryos, when compared to miR-2188 duplex injected embryos. B) Embryo lysates were prepared from 24 hpf embryos injected with the GFP reporter in the presence or absence of MO<sub>miR-2188</sub>, miR-2188 and scrambled duplexes or a mixture of MO<sub>miR-2188</sub> and miR-2188. Protein levels were determined by western blot analysis and β-tubulin was used as an internal control. C) Quantification of the reporter proteins (%). Asterisks indicate conditions where GFP expression was significantly down regulated by miR-2188 relative to control conditions. miR-2188 injected embryos showed a statistically significant down regulation of GFP levels, indicating that *Nrp2a* 3'UTR is a true miR-2188 target. Co-injection of the MO<sub>miR-2188</sub> with the miR-2188 duplex resulted in the rescue of GFP fluorescence, further confirming that *Nrp2a* is a bona fide target of miR-2188. Data are mean ± stdev, p<0.005 (t test, unpaired), n>3. All lanes were normalized to the β-tubulin signal. doi:10.1371/journal.pone.0039417.g005

be zebrafish specific. This is in line with the hypothesis that recently evolved and non-conserved miRNAs may regulate species specific genes and may play a key role in evolutionary processes [39].

In conclusion, our data shows for the first time that miR-2188 targets *Nrp2a*, a positive regulator of angiogenesis, indicating that the VEGF pathway can be regulated by miRNAs controlling VEGF co-receptors.

## Materials and Methods

### Ethics Statement

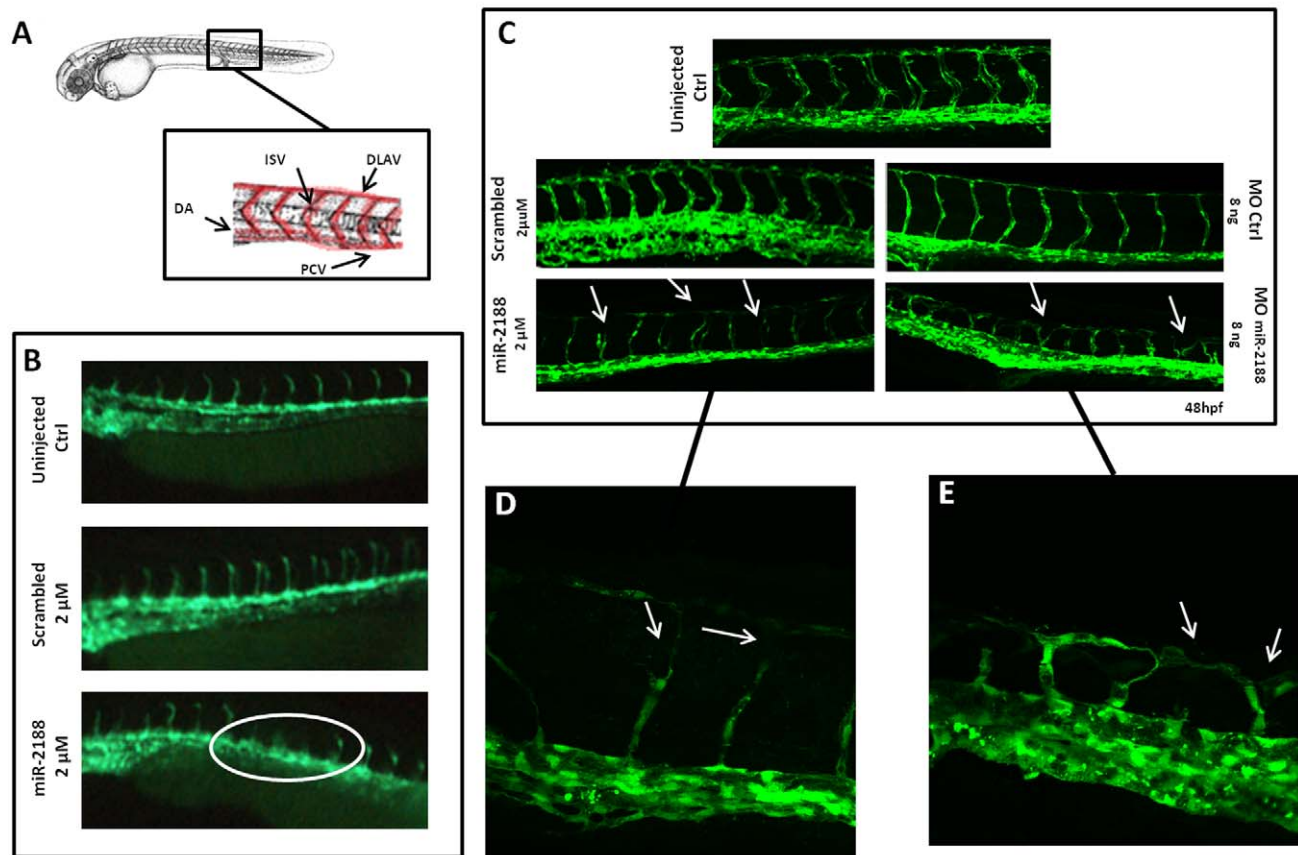
All experiments were performed according to the European law for animal experiments (2010/63/EU). No specific ethics approval under EU guidelines was required for this project, as all zebrafish used in this study were between 0 and 5 days old. This is within the European law (Council Directive 86/609/EEC), which excludes foetal and embryonic forms.

Wild type AB and Tg(flk1-GFP)s843 zebrafish were maintained under the protocols approved by the Institutional Animal Ethics Committee of the University of Liège under application number 567. Zebrafish embryos were collected and kept at 28°C under standard laboratory conditions, and staged as described [40].

### miRNA Duplex Injections

A miR-2188 RNA duplex and a duplex containing a scrambled sequence were obtained as siRNAs from SIGMA:

miR-2188: sense 5'-AAGGUCCAACCUCACAUGUCC<sub>RNA</sub>-3';  
 antisense 5'-ACAUGUGAGGUUGGACCU<sub>RNA</sub>[dT][dT]-3';  
 scrambled: sense 5'-GUGUACACGUCUAUACGCC-CA<sub>RNA</sub>-3';  
 antisense 5'-GGCGUAUAGACGUGUACAC<sub>RNA</sub>[dT][dT]-3'; and were injected into one-cell fertilized embryos at 2 μM.



**Figure 6. Analysis of blood vessel formation in *Tg(flk1-GFP)s843* embryos.** A) Representation of the zebrafish circulatory system showing the major structures (DLAV - Dorsal Longitudinal Anastomotic Vessel; ISV - Intersegmental Vessel; DA - Dorsal Aorta; PCV - Posterior Cardinal Vein). B) Visualization of 24 hpf embryo blood vessels using fluorescence microscopy. After miR-2188 duplex microinjection under developed and absent ISVs were observed, non injected and scrambled duplex injected embryos did not show such defects. C) Visualization of 48 hpf embryo blood vessels using confocal microscopy (20x). ISVs of miR-2188 injected embryos were thinner than those of non injected and scrambled duplex injected embryos and displayed ISV's patterning defects (arrows). MO<sub>miR-2188</sub> injected embryos revealed DLAV defects and branching of ISVs (arrows). Images shown in D) and E) are 60x amplification images of miR-2188 duplex and miR-2188-MO injected embryos, respectively.  
doi:10.1371/journal.pone.0039417.g006

Approximately 1000 pL of 2  $\mu$ M (miR-2188 and scrambled duplex) diluted in TE buffer and 5% Rhodamine were injected in one cell embryos. Embryos were staged and examined for phenotypic alterations.

### Morpholino Injections

The miR-2188 morpholino (MO) was obtained from Gene Tools, diluted in Daniaeu Buffer and 5% Rhodamine and 1000 pL were injected in one cell embryos at 8 ng/ $\mu$ L. 1000 pL of a control MO from Gene Tools was also injected at 8 ng/ $\mu$ L. Phenotypes were assessed at 24 hpf and after blood circulation was established.

### Image Acquisition

24 hpf and 48 hpf control and injected (either with miRNA duplexes or MOs) *Tg(flk1-GFP)s843* embryos were dechorionated and fixed in 4% PFA at 4°C for 4 h, washed 3 times in PBS-T and stored at 4°C. For imaging, stored embryos were mounted in Prolong and visualized using an OLYMPUS FV1000 confocal microscope, equipped with FV-10ASW software, FITC filters and a 488 nm laser. Images from control and injected embryos with the duplexes or the MOs were acquired with 20x, 40x and 60x objectives.

### Quantitative Real-time PCR

Total RNA was extracted from embryos injected with the miRNA duplexes, from embryos injected with miRNA MOs and from control embryos at 48 hpf using Qiazol (Qiagen), following the manufacturer's protocol. Samples were treated with DNase to remove any contaminating genomic DNA. RNA quantity and quality were assessed using the Nanodrop and Agilent 2100 bioanalyzer systems, respectively. Samples with a RNA integrity number (RIN) above 7 were used in this study.

Quantification of miR-2188 expression was carried out using NCode<sup>TM</sup> miRNA first-strand cDNA module and Platinum<sup>®</sup> SYBR<sup>®</sup> Green qPCR Super Mix-UDG from Invitrogen. Briefly, miRNAs were polyadenylated using poly-A polymerase and ATP. cDNA was then prepared from 500 ng of total RNA with the SuperScript<sup>®</sup> III RT and a Universal RT primer, following the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed with the synthesized cDNA using SYBR<sup>®</sup> Green detection reagent, the Universal qPCR primer provided in the kit, and a forward primer designed to target miR-2188 (5'-AAGGTC-CAACCTCACATGTCC-3'). U6 small RNA was used as an endogenous control for normalizing miRNA levels.

Quantification of *Nrp2a* was carried out using Power SYBR<sup>®</sup> Green PCR Master Mix from Applied Biosystems. cDNA was



**Table 2.** Putative targets of dre-miR-2188.

Gene ID (ZFIN)	Gene name	GO Molecular Function	GO Biological process	GO Cellular component
Nfe2l1	nuclear factor, erythroid derived 2,-like 1a	Binding: DNA	Regulation of transcription	Nucleus
Has2	hyaluronan synthase 2	Hyaluronan synthase activity; Transferase activity, transferring hexosyl groups	atrioventricular valve development; cell migration in hindbrain; cell migration involved in gastrulation; dorsal convergence; embryonic heart tube development; heart looping; hyaluran biosynthetic process; mesodermal cell migration; metabolic process; somitogenesis	Membrane
Myst3	K(lysine) acetyltransferase 6A	DNA binding; transferase activity; zinc ion binding	anterior/posterior pattern specification; cartilage development; embryonic body morphogenesis; embryonic pattern specification; embryonic skeletal system development; embryonic viscerocranium morphogenesis; histone acetylation; neural crest cell fate specification; nucleosome assembly; regulation of transcription, DNA-dependent	Nucleus; Nuclear chromatin; Nucleosome;
Cpla2	cytosolic phospholipase a2	hydrolase activity; lysophospholipase activity; metal ion binding; phospholipase activity	lipid catabolic process; metabolic process; ovarian follicle development; phospholipid catabolic process	cytoplasm; cytoplasmic membrane-bounded vesicle; cytoplasmic vesicle
Atg13	Autophagy related protein 13	molecular	autophagy	cytoplasm; cytosol; pre-autophagosomal structure
Dnajc3	DnaJ (Hsp40) homolog, subfamily C, member 3	binding; heat shock protein binding; unfolded protein binding;	protein folding	cellular component
Dnaja3a	DnaJ (Hsp40) homolog, subfamily A, member 3A	ATP binding; heat shock protein binding; metal ion binding; unfolded protein binding	protein folding; response to heat	unknown
Pdik1l	PDLIM1 interacting kinase 1 like	ATP binding; kinase activity; nucleotide binding; protein kinase activity; protein serine/threonine kinase activity; transferase activity; transferase activity, transferring phosphorus-containing groups	phosphorylation; protein phosphorylation	nucleus

doi:10.1371/journal.pone.0039417.t002

prepared from 500 ng of total RNA with the SuperScript<sup>®</sup>II RT (Invitrogen) following the manufacturer's instructions. qPCR was performed with the synthesized cDNA using Power SYBR<sup>®</sup> Green detection reagent and forward (5'-TGGAGCTACTCTGTTTC-CAGC-3') and reverse (5'- TGTCCAAAGAGAGACTGGGA-3') *Nrp2a* primers. Tubulin  $\alpha$ -1 was used as an internal control for normalizing mRNA levels as its expression level is relatively constant across tissues and it is not significantly altered by experimental conditions [41].

Efficiency of primers was determined after the generation of a standard curve. All reverse transcriptions and no-template controls were carried out simultaneously following the RT step. qPCR was

carried out using the ABI Prism 7500 Sequence Detector System (Applied Biosystems). Reactions were incubated in 96-well optical plates and cycling began with template denaturation at 95°C for 2 min, followed by 50 cycles at 95°C for 15 seconds and 60°C for 35 seconds for miR-2188 detection. For *Nrp2a* detection, cycling began with template denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min. The threshold cycle data (CT) and baselines were determined using auto settings. All assays including no template controls were carried out in triplicate. The REST software [42] was used to quantify *Nrp2a* and miRNA levels. In our dataset the control

samples were the scrambled (for duplex injection) or MO<sub>ctrl</sub> (for MO injections).

### In Situ Hybridization

*In situ* hybridization was carried out as described [43] with a *Nrp2a* probe kindly provided by the Laboratory of Molecular Biology and Genetic Engineering, Liège University. After staining, embryos were washed 4 times with TBS-T. Embryos were mounted in 100% glycerol and photographed using a color digital camera.

miR-2188 ISH was carried out with a LNA probe from Exiqon (/5DigN/GGACATGTGAGGTTGGACCTT/3Dig\_N/, Tm 79°C). Briefly, 24 hpf and 48 hpf embryos were collected and prepared for ISH as described above. Incubations with hybridization mix and miR-2188 probe were performed at 61°C to minimize unspecific hybridization. 10 nM of miR-2188 probe were used for the ISH.

### Sensor assay

The pCS2+ plasmid containing eGFP (gift from Dr. Hyangshuk Rhim, Research Institute of Molecular genetics, Catholic University of Korea), was used in this study. The 3'UTR of *Nrp2a* containing putative miR-2188 binding sites was fused to the 3' end of eGFP using StuI and XhoI. The 3' UTR of *Nrp2a* was amplified using the following primers:

Forward: 5'-GCGCAGGCCTTCCTAAAATGACCT-CAAAGT-3'.

Reverse: 5'-GCGCCTCGAGGTATATCGAGTGTTTAA-GAC-3'.

*Nrp2a* 3'UTR sequences carrying point mutations in the predicted miR-2188 binding sites were engineered by site directed mutagenesis. A M1 construct containing mutations in the binding site-1 (BS-1) was generated using the following primers: Forward: 5'-GGGAGATCTGTGGCCGGGACCCCGAGCCAT-GAACTTCTGTCC-3' Reverse: 5'-GGACAGAAGTT-CATGGCTCGGGGTCCCGGCCACAGATGTCCC-3'.

A M2 construct containing the mutation in the binding site-2 (BS-2) was generated with the following primers:

Forward: 5'-TTTGCAAACGGGTTAGTGCTGAACACT-GAGCCTTGAGG-3'.

Reverse: 5'-CCTCAAGGCTCAGTGTTCAGCAC-TAACCCGTTTGCAAA-3'.

Highlighted nucleotides show the mutations inserted in the 3'UTR. Point mutations were PCR amplified and fused to the 3' end of eGFP, as described above. For all constructs, capped mRNA was prepared to avoid degradation after microinjection, using the mMESSAGE mMACHINE SP6 Kit (Ambion), according to manufacturer's protocol. The mRNA (final concentration 50 ng/μL) was injected with either miR-2188 duplex or the control miRNA duplex containing a scrambled miRNA sequence (final concentration 2 μM). Approximately 1000 pL of the mixture of mRNA and duplex were injected and embryos were pooled and prepared for western blot analysis at 24 hpf.

### References

- Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281–297.
- Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* 9: 102–114.
- He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 5: 522–531.
- Kim VN, Han J, Siomi MC (2009) Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* 10: 126–139. nrm2632 [pii];10.1038/nrm2632 [doi].
- Chekulaeva M, Filipowicz W (2009) Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol* 21: 452–460. S0955-0674(09)00095-7 [pii];10.1016/j.ceb.2009.04.009 [doi].
- Rajewsky N (2006) microRNA target predictions in animals. *Nat Genet* 38 Suppl: S8–13. ng1798 [pii];10.1038/ng1798 [doi].
- Giraldez AJ, Cinalli RM, Glasner ME, Enright AJ, Thomson JM, et al (2005) MicroRNAs regulate brain morphogenesis in zebrafish. *Science* 308: 833–838.
- Mishima Y, Abreu-Goodger C, Staton AA, Stahlhut C, Shou C, et al (2009) Zebrafish miR-1 and miR-133 shape muscle gene expression and regulate sarcomeric actin organization. *Genes Dev* 23: 619–632. gad.1760209 [pii];10.1101/gad.1760209 [doi].

### Western Blot

24 hpf embryos were pooled (20–30 embryos each) and embryo extracts were prepared after dechoriation and devolking. 2xSDS sample buffer was added, followed by denaturation at 95°C for 5 min followed by vortexing. The protein solution was centrifuged for 2 min using a table-top centrifuge and samples were directly loaded onto 15% PAA protein gels and electrophoresed in SDS running buffer. Proteins were transferred overnight at 4°C to PVDF Hybond-P membranes (GE Healthcare), which were blocked for 1 h with 4% nonfat dry milk in TBS-T. Immunodetection was carried out using monoclonal anti-Green Fluorescent Protein mouse antibody (1:1000) from Clontech and anti β-tubulin mouse antibody (1:500) from Invitrogen, as primary antibodies. IRDye®800 CW Goat anti-mouse IgG from LI-COR® was used as secondary antibody (1:10000). Detection was carried out using Odyssey® Imaging System.

### Supporting Information

**Figure S1 Developmental defects observed after miR-2188 duplex and Mo<sub>miR-2188</sub> injections.** At 48 hpf developmental defects were quantified. Scrambled and MO<sub>ctrl</sub> injected embryos developed similarly to non-injected embryos, while embryos injected with the miR-2188 duplex revealed high incidence of pericardial edema (70%) and circulation arrest (50%). More than 50% of the embryos lacked one or more ISVs. MO<sub>miR-2188</sub> injected embryos showed ISV branching (50%) that often resulted in dorsal longitudinal anastomotic vessel (DLAV) defects (50%). Twenty four embryos were analyzed per condition (n>3). (TIFF)

**Figure S2 ISV patterning is rescued after injection of Mo<sub>miR-2188</sub> in embryos overexpressing miR-2188.** Visualization of 48 hpf embryo blood vessels using confocal microscopy (20x). ISVs of miR-2188 injected embryos were thinner and displayed ISV patterning defects, when compared to non-injected embryos. Injection of MO<sub>miR-2188</sub> in embryos over expression miR-2188 rescued the ISV patterning. (TIFF)

### Acknowledgments

We thank Dr. Hyangshuk Rhim from the Research Institute of Molecular genetics, Catholic University of Korea for kindly providing the pCS2+ plasmid containing eGFP.

### Author Contributions

Conceived and designed the experiments: ARS PP HP IS MS. Performed the experiments: ARS MR ON AB. Analyzed the data: ARS PP HP MS. Contributed reagents/materials/analysis tools: AB GM. Wrote the paper: ARS PP HP MS.

9. Tay YMS, Tam WL, Ang YS, Gaughwin PM, Yang H, et al (2008) MicroRNA-134 modulates the differentiation of mouse embryonic stem cells, where it causes post-transcriptional attenuation of Nanog and LRH1. *Stem Cells* 26: 17–29.
10. Flynt AS, Thatcher EJ, Burkewitz K, Li N, Liu Y, et al (2009) miR-8 microRNAs regulate the response to osmotic stress in zebrafish embryos. *J Cell Biol* 185: 115–127. doi:10.1083/jcb.200807026 [pii];10.1083/jcb.200807026 [doi].
11. Li N, Wei C, Olena AF, Patton JG (2011) Regulation of endoderm formation and left-right asymmetry by miR-92 during early zebrafish development. *Development* 138: 1817–1826. doi:10.1242/dev.056697 [pii];10.1242/dev.056697 [doi].
12. Care A, Catalucci D, Felicetti F, Bonci D, Addario A, et al (2007) MicroRNA-133 controls cardiac hypertrophy. *Nat Med* 13: 613–618.
13. Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, et al (2009) MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc Natl Acad Sci U S A* 106: 2735–2740. doi:10.1073/pnas.0811073106 [pii];10.1073/pnas.0811073106 [doi].
14. Silber J, Lim DA, Petritsch C, Persson AI, Maunakea AK, et al (2008) miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med* 6: 14.
15. Soares AR, Pereira PM, Santos B, Egas C, Gomes AC, et al (2009) Parallel DNA pyrosequencing unveils new zebrafish microRNAs. *Bmc Genomics* 10: ARTN 195;DOI 10.1186/1471-2164-10-195.
16. Geretti E, Klagsbrun M (2007) Neuropilins: novel targets for anti-angiogenesis therapies. *Cell Adh Migr* 1: 56–61. doi:10.1007 [pii].
17. Kawasaki T, Kitsukawa T, Bekku Y, Matsuda Y, Sanbo M, et al (1999) A requirement for neuropilin-1 in embryonic vessel formation. *Development* 126: 4895–4902.
18. Lee P, Goishi K, Davidson AJ, Mannix R, Zon L, et al (2002) Neuropilin-1 is required for vascular development and is a mediator of VEGF-dependent angiogenesis in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America* 99: 10470–10475. doi:10.1073/pnas.162366299.
19. Martyn U, Schulte-Merker S (2004) Zebrafish neuropilins are differentially expressed and interact with vascular endothelial growth factor during embryonic vascular development. *Developmental Dynamics* 231: 33–42. doi:10.1002/dvdy.20048.
20. Carmeliet P (2005) Angiogenesis in life, disease and medicine. *Nature* 438: 932–936. doi:10.1038/nature04478 [pii];10.1038/nature04478 [doi].
21. Favier B, Alam A, Barron P, Bonnin J, Laboudie P, et al (2006) Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. *Blood* 108: 1243–1250. doi:10.1182/blood-2005-11-4447 [pii];10.1182/blood-2005-11-4447 [doi].
22. Zhang S, Zhou HE, Osunkoya AO, Iqbal S, Yang X, et al (2010) Vascular endothelial growth factor regulates myeloid cell leukemia-1 expression through neuropilin-1-dependent activation of c-MET signaling in human prostate cancer cells. *Mol Cancer* 9: 9. doi:10.1186/1476-4598-9-9 [pii];10.1186/1476-4598-9-9 [doi].
23. Shen J, Samul R, Zimmer J, Liu H, Liang X, et al (2004) Deficiency of neuropilin 2 suppresses VEGF-induced retinal neovascularization. *Mol Med* 10: 12–18.
24. Bovenkamp DE, Goishi K, Bahary N, Davidson AJ, Zhou Y, et al (2004) Expression and mapping of duplicate neuropilin-1 and neuropilin-2 genes in developing zebrafish. *Gene Expression Patterns* 4: 361–370. doi:10.1016/j.modgep.2004.01.014.
25. Kim WH, Lee SH, Jung MH, Seo JH, Kim J, et al (2009) Neuropilin2 expressed in gastric cancer endothelial cells increases the proliferation and migration of endothelial cells in response to VEGF. *Exp Cell Res* 315: 2154–2164. doi:10.1016/j.yexcr.2009.04.018 [pii];10.1016/j.yexcr.2009.04.018 [doi].
26. Wang S, Olson EN (2009) AngiomiRs—key regulators of angiogenesis. *Curr Opin Genet Dev* 19: 205–211. doi:10.1016/j.gde.2009.04.002 [pii];10.1016/j.gde.2009.04.002 [doi].
27. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, et al (2009) MicroRNA-92a Controls Angiogenesis and Functional Recovery of Ischemic Tissues in Mice. *Science* 324: 1710–1713. doi:10.1126/science.1174381.
28. Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, et al (2008) miR-126 regulates angiogenic signaling and vascular integrity. *Dev Cell* 15: 272–284. doi:10.1016/j.devcel.2008.07.008 [pii];10.1016/j.devcel.2008.07.008 [doi].
29. Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, et al (2008) The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell* 15: 261–271. doi:10.1016/j.devcel.2008.07.002 [pii];10.1016/j.devcel.2008.07.002 [doi].
30. Wurdinger T, Tannous BA, Saydam O, Skog J, Grau S, et al (2008) miR-296 regulates growth factor receptor overexpression in angiogenic endothelial cells. *Cancer Cell* 14: 382–393. doi:10.1016/j.ccr.2008.10.005 [pii];10.1016/j.ccr.2008.10.005 [doi].
31. Stark A, Brennecke J, Bushati N, Russell RB, Cohen SM (2005) Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3'UTR evolution. *Cell* 123: 1133–1146. doi:10.1016/j.cell.2005.11.023 [pii];10.1016/j.cell.2005.11.023 [doi].
32. Flynt AS, Thatcher EJ, Burkewitz K, Li N, Liu YZ, et al (2009) miR-8 microRNAs regulate the response to osmotic stress in zebrafish embryos. *Journal of Cell Biology* 185: 115–127. doi:10.1083/jcb.200807026.
33. Leucht C, Stigloher C, Wizenmann A, Klafke R, Folchert A, et al (2008) MicroRNA-9 directs late organizer activity of the midbrain-hindbrain boundary. *Nature Neuroscience* 11: 641–648. doi:10.1038/nn.2115.
34. Zhao Y, Srivastava D (2007) A developmental view of microRNA function. *Trends Biochem Sci* 32: 189–197. doi:10.1016/j.tibs.2007.02.006 [pii];10.1016/j.tibs.2007.02.006 [doi].
35. Evangelisti C, Florian MC, Massimi I, Dominici C, Giannini G, et al (2009) MiR-128 up-regulation inhibits Reelin and DCX expression and reduces neuroblastoma cell motility and invasiveness. *FASEB J* 23: 4276–4287. doi:10.1096/fj.09-134965 [pii];10.1096/fj.09-134965 [doi].
36. Herbert SP, Odell AF, Ponnambalam S, Walker JH (2009) Activation of cytosolic phospholipase A2- $\alpha$  as a novel mechanism regulating endothelial cell cycle progression and angiogenesis. *J Biol Chem* 284: 5784–5796. doi:10.1074/jbc.M807282200 [pii];10.1074/jbc.M807282200 [doi].
37. Alberghina M (2010) Phospholipase A(2): new lessons from endothelial cells. *Microvasc Res* 80: 280–285. doi:10.1016/j.mvr.2010.03.013 [pii];10.1016/j.mvr.2010.03.013 [doi].
38. Griffiths-Jones S, Saini HK, van DS, Enright AJ (2008) miRBase: tools for microRNA genomics. *Nucleic Acids Res* 36: D154–D158.
39. Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, et al (2005) Identification of hundreds of conserved and nonconserved human microRNAs. *Nat Genet* 37: 766–770. doi:10.1038/ng1590 [pii];10.1038/ng1590 [doi].
40. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* 203: 253–310. doi:10.1002/aja.1002030302 [doi].
41. McCurley AT, Callard GV (2008) Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Mol Biol* 9: 102. doi:10.1186/1471-2199-9-102 [pii];10.1186/1471-2199-9-102 [doi].
42. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30: e36.
43. Thisse C, Thisse B (2008) High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc* 3: 59–69. doi:10.1038/nprot.2007.514 [pii];10.1038/nprot.2007.514 [doi].